Beta 2 Tubulin Amino Acids Required for Sperm Tail Axoneme Function

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Abstract

Evolutionary changes in organism traits are primarily caused by random genetic mutations in their amino acid codons that end up altering the proteins produced. The main question of researchers is how changes occur that will give a protein a new function without detrimentally affecting the original function of the protein in the organism. Beta 2 tubulin in Drosophila is an ideal model to study this question because it has a very sensitive structure/function relationship. Drosophila contains two main types of tubulin: Beta 1 which is found in the majority of cells and testes specific Beta 2. These proteins differ in only a few amino acids, however Beta 1 is unable to support the function of Beta 2. The proposed continues study of what allows Beta 2 to make a spermtail when Beta 1 cannot. I will investigate a synergistic interaction between amino acids 29, 55, and 57 of the testes specific Beta 2 tubulin protein in Drosophila by exchanging Beta 1 codons with Beta 2 identity at these sites to generate a chimeric Beta 1-Beta 2 tubulin (TGARC). The ability of TGARC to support spermtail axoneme function will be determined through fertility studies, protein expression analysis, sperm tail length comparisons, and axoneme cross sectional comparisons using TEM.

Introduction

Though the course of history, organisms have been evolving by a single primary means, referred to as evolution by natural selection. The evolutionary syllogism contains two premises that result in evolution:

1) If there is heritable variation in phenotype
2) There will be a competition among variants for resources, with the most competitive variants passing their genotypes to the next generation

This results in evolution.

The first premise addresses the availability of choices in particular features based upon genetic mutations in the organism's DNA sequence. Some features, including proteins, may admit of change more readily than others, and this will influence the evolutionary process. The second premise addresses the competition among organisms provided with different phenotypes for the occupation of particular ecological niches. However competition would not be possible if phenotypic choices had not been available with which to compete with. It is generally accepted that evolution is the force behind the generation of new species; however the exact roles of each premise are not fully understood.

Researchers are trying to find out how proteins change in an organism to gain a new function without altering the old function. Part of the answer to this inquiry is gene duplication. When a particular gene duplicates, it provides a basis for evolution by allowing one copy of the gene to continue performing its desired function, while the other copy of the gene is free to change into a new function. *Drosophila Beta 2 tubulin* is an excellent model for this study, because previous research indicates this route must be a very narrow path given every small change in Beta 2 thus far examined does not maintain the function of the protein, a result reflected in the 60 million year stasis of the protein in Drosophilids.

Evolutionary change in proteins occurs due to alterations in the amino acid coding sequences of an organism's genetic code, which results in the alteration of proteins. Not all features or proteins are equally variable; some are more easily altered than others. Here we seek to determine why Beta 2 protein does not admit of heritable variation, by testing if there is a unique Beta 2 amino acid synergism that is fundamental to Beta 2 function that, due to being a synergism, is resistant to evolutionary change.

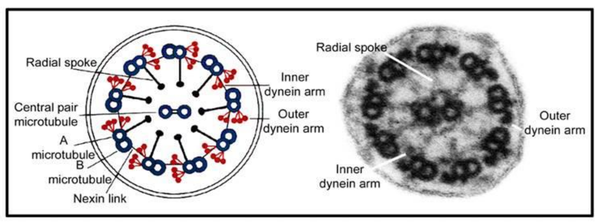

Fig. Proper structure of sperm tail axoneme

DmB1 MREIVHIQAG QCGNQIGAKF WEIISDEHGI DATGAYHGDS DLQLERINVY YNEASGGKYV PRAVLVDLEP GTMDSVRSGP [80]
DmB2 MREIVHIQAG QCGNQIGKF WEVISDEHCI DATGTYYGDS DLQLERINVY YNEATGAKYV PRAILVDLEP GTMDSVRSGA [80]

DmB1 FGQIFRPDNF VFGQSGAGNN WAKGHYTEGA ELVDSVLDVV RKEAESCDCL QGFQLTHSLG GGTGSGMGTL LISKIREEYP [160]
DmB2 FGQIFRPDNF VFGQSGAGNN WAKGHYTEGA ELVDSVLDVV RKESEGCDCL QGFQLTHSLG GGTGSGMGTL LISKIREEYP [160]

DmB1 DRIMNTYSVV PSPKVSDTVV EPYNATLSVH QLVENTDETY CIDNEALYDI CFRTLKLTTP TYGDLNHLVS LTMSGVTTCL [240]
DmB2 DRIMNTFSVV PSPKVSDTVV EPYNATLSVH QLVENTDETY CIDNEALYDI CFRTLKLTTP TYGDLNHLVS ATMSGVTTCL [240]

DmB1 RFPGQLNADL RKLAVNMVPF PRLHFFMPGF APLTSRGSQQ YRALTVPELT QQMFDAKNMM AACDPRHGRY LTVAAIFRGR [320]
DmB2 RFPGQLNADL RKLAVNMVPF PRLHFFMPGF APLTSRGSQQ YRALTVPELT QQMFDAKNMM AACDPRHGRY LTVAAIFRGR [320]

DmB1 MSMKEVDEQM LNIQNKNSSY FVEWIPNNVK TAVCDIPPRG LKMSATFIGN STAIQELFKR ISEQFTAMFR RKAFLHWYTG [400]
DmB2 MSMKEVDEQM LNIQNKNSSF FVEWIPNNCK TAVCDIPPRG LKMSATFIGN STAIQELFKR VSEQFTAMFR RKAFLHWYTG [400]

DmB1 EGMDEMEFTE AESNMNDLVS EYQQYQEATA DEDAEFEEEQ EAEVDEN* [448]
DmB2 EGMDEMEFTE AESNMNDLVS EYQQYQEATA DEEGEFDEDE EGGGDE-* [448]

Fig. 1. Alignment of Beta 1 and Beta 2 proteins. Underlined/red characters show the amino acid differences between the two proteins.

Background

Drosophila beta-tubulin is a prime example to study the first premise of the Darwinian syllogism by examining what prevents this protein from evolving in *Drosophila*. *Drosophila* has Beta 1 tubulin found in almost all of their cells, and they have Beta 2 tubulin found only in their sperm tail axoneme. Their sperm tail is exceptionally long, 2 mm, and may require a specialized tubulin to support this length.

Beta 1 is 95% similar to Beta 2 and differs in amino acid sequence by only 25 amino acids (Fig. 1). Previous research has shown that Beta 1 alone could not replace Beta 2 in sperm tail functioning; Beta 2 is in fact specialized in its ability to support the sperm tail. The primary question is why can Beta 2 provide a functional sperm tail axoneme when Beta 1 cannot? Previous work tested the function of the Beta 2 carboxy terminus, amino acids 381-446, by exchanging them into the Beta 1 protein. It could not replace Beta 2 function; however sperm tail length was closer to that of wild type flies indicating that these residues carried their function into the Beta 2 protein in an additive manner. However, Beta 1 with Beta 2 amino acids at locations 55 and 57 along with the Beta 2 carboxyl tail lost Beta 2 function, revealed in its generating a much shorter sperm tail. This indicates that the proper function of 55 and 57 depends on Beta 2 specific amino acid interactions – a synergism. Protein crystallography of Beta 2 showed that in the folded protein, amino acids 55 and 57 directly abut amino acid 29 (Fig. 2), and phylogenetic analyses show that the Beta 2 amino acid identities at these sites are unique among the more than 100 beta tubulins . This information led to the hypothesis that there was a synergistic interaction between Beta 2 amino acids 55, 57, and 29, providing a unique Beta 2 motif that would not function without having all the correct amino acids in place to complete the synergism. Study of this unique synergistic interaction is the main focus of this research project.

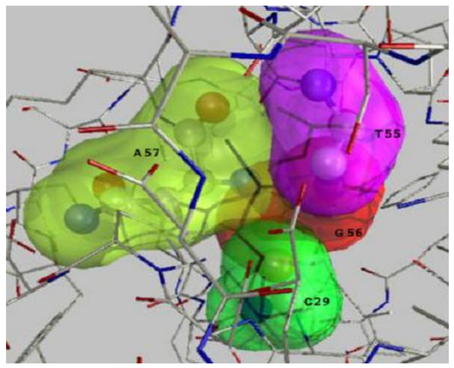

Fig. 2. Amino acids 29, 55, 56,57 in the folded tubululn protein

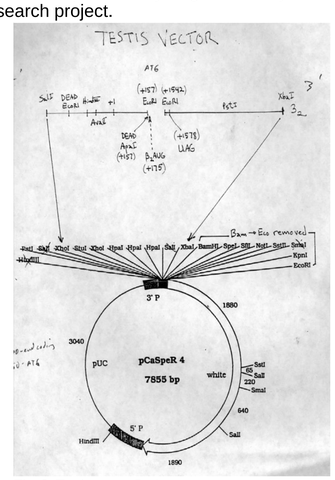

Fig. 3. Testis vector P-element construct

Methods

A p-element construct (Fig. 3.) containing Beta 1-tubulin along with Beta 2 codons at locations 29, 55, 57, and 381-446 was designed and designated *TGARC*. The *TGARC* construct was sent to an offsite location (Rainbow Transgenics, CA) to be transformed into W1118 (white -) flies. The p-element vector contained the white+ gene that generates a red eyed phenotype, so the transformed flies containing the *TGARC* insert could be distinguished due to the expression of a red eyed phenotype.

To ensure that *TGARC* was the sole source of beta tubulin in the testes, specific crosses were done using a "K" designated chromosome that contains a null copy of *Beta 2 tubulin*. Virgin flies containing the *TGARC* insert were mated with a stock of opposite sex K/TM3 flies (TM3 is a balancer chromosome with a wildtype copy of *Beta 2)* in order to generate *TGARC* K/TM3 flies. K has the genotype radius incompletus (ri), Beta 2-, and ebony. TM3 has the genotype short bristles (Sb), ri, and ebony. Genotypes can be distinguished because flies containing short bristles were known to be of the genotype K/TM3, which could be distinguished from those flies with the K/K genotype that contained long bristles. The progeny of these crosses expressing the red eye mutant phenotype were again crossed to produce flies that would be homozygous for *TGARC*/*TGARC* and Beta2-/Beta2- in the testes.

Some of the *TGARC*/*TGARC* and Beta2-/Beta2- progeny found with the desired genotype were mated with the stock K/TM3 flies to test for fertility of the mutant tubulin in the testes. Others were set aside for testes dissections in order to look at sperm tail length, mobility, and meiotic/mitotic activity in the testes using phase contrast microscopy and protein expression using protein acrylamide gel electrophoretic analysis. Each dissection was performed under a dissection microscope in TBI buffer using forceps to remove all extraneous tissue, leaving just the testes pair for further examination.

Testes dissections were performed in TBI on 2-3 day old virgin male flies with genotypes *white-/y; +/+; B2-/B2+, white-/y; +/+; B2-/B2-, and white+/y; TGARC/TGARC; B2-/B2-* so that there were eight testes for each genotype. Once eight testes for a particular genotype had been collected, they were added to aliquots of Laemmli sample buffer and boiled. Samples were washed in acetone and spun to acquire a protein pellet. Pellets were resuspended and run on SDS page gels following standard Western Blotting technique. Antibody blocking was used to view protein bands on developed film.

Results

A light microscope was used to look at the dissected testes of the flies with genotypes *white-/y; +/+; B2-/B2+, white-/y; +/+; B2-/B2-, and white+/y; TGARC/TGARC; B2-/B2-*. As previously mentioned, only the flies with a red eyed phenotype contain the *white+* gene, and therefore the linked *TGARC* gene. The white eyed flies were used as positive and negative controls to compare to *TGARC/TGARC* testes. The white eyed *B2-/B2+* flies were found to be completely normal. The testes were loaded with fully formed and fully functioning sperm (Fig. 5a). As expected, the white eyed *B2-/B2-* flies contained no sperm because each fly of this phenotype has two copies of Beta 2-, and is therefore unable to produce sperm (Fig. 5b). The red eyed *TGARC/TGARC; B2-/B2-* flies were not what was expected. The *TGARC* insert was expected to provide these flies with a means to produce some resemblance of sperm despite having two copies of *Beta 2-*. However, no sperm were found and these testes very closely resembled those of their white eyed counterpart. Some pre-meiotic spermatids were seen attempting to undergo meiosis and form sperm (Fig. 5b), but they were unable to complete the process.

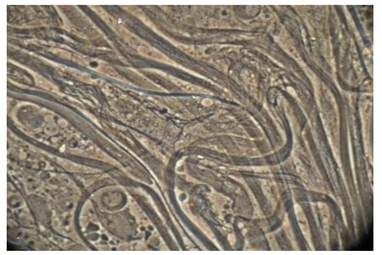

Fig. 5a. Cysts each containing 64 wildtype haploid sperm

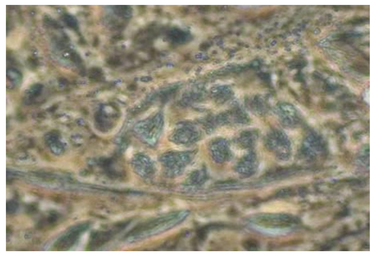

Fig. 5b. Cyst with 16 pre-meiotic spermatids showing failure of meiosis and sperm tail elongation.

Red eyed K/K male flies were crossed with a virgin female white eyed K/T wild-type fly to test for the potential fertility of the red eyed flies containing the *TGARC* vector. None of the mating pairs produced anything more than eggs in their culture tubes.

Testes from *white-/y; +/+; B2-/B2+*, (white eyed K/T), *white-/y; +/+; B2-/B2-*, (white eyed K/K), and *TGARC / TGARC B2-/B2-*(red eyed K/K flies) were run on a western protein gel to determine if there was a difference in the Beta proteins present between each of the three test phenotypes. The gel showed that there was no apparent difference between the *TGARC/TGARC; B2-/B2-* and *B2-/B2-* flies, neither of which showed the beta tubulin band found in the *B2-/B2+* flies.

Primers designed to amplify the *TGARC* gene in our p-element vector were used in conjuncture with PCR to determine if *TGARC* was present in *white+* transformed flies. None of the transformed flies showed *TGARC* amplification. *TGARC* vector DNA was used as a positive control, which did amplify indicating the primers work.

Discussion

The largest surprise from the results obtained was the complete lack sperm found in the testes of *TGARC/TGARC* flies. Based on previous results from other tests of Beta 2 amino acids in a Beta 1 background, some resemblance of sperm should have been found, regardless of whether or not they were full length or motile. The whole hypothesis for the *TGARC* vector was based on previous work that had been slowly increasing the length and improving the structure of the axoneme until the potential synergism was found. We believed we would see improved function in spermatogenesis, and certainly expected some spermatogenic function, given prior tests of chimeric tubulins.

The gel electrophoresis and the PCR test confirmed that the flies did not contain the *TGARC* insert. What is surprising about this is that *TGARC* was linked in the vector to *white+*. However, it appears that the white + gene that gives the flies their red eye color was transformed in the flies without *TGARC*. At this time, there is no feasible hypothesis to how this could have occurred other than the *TGARC* sequence may not have been transformed into the vector. The next step is to check the vector itself for the presence of *TGARC* by sequencing and then move to reinject new flies with the vector.

Despite the problem with the *TGARC* gene transformation, our hypothesis was still a good one that could yield promising results once the transformation issue is resolved. The most promising possibility would be that the addition of Beta 2 amino acid 29 would in fact complete the synergism allowing the creation of structurally and functionally complete sperm axonemes. In this case, it would be expected that these flies would be fertile and able to reproduce normally.

Another possibility could be that the addition of amino acid 29 improves the sperm tail somewhat, but not completely. In this case, there would still be a piece missing that would be necessary to reach full functionality. The sperm would not be fertile and would most likely be still, however the tail length would be closer to that of the wild type flies and the correct structure of microtubules would be present.

A final possibility to occur from the addition of amino acid 29 would be another regression of structure and function. The promising prospect of completing the synergism with this additional amino acid may actually worsen the protein. If this is the case, the flies may not be able to produce sperm at all, just like what we have seen so far. Another possibility is that sperm would be created, but would be much shorter than the wild type, or the structure of microtubules in the axoneme could be completely wrong.